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Effects of Phyto-Oestrogens on Veal Calf Prostate Histology

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ABSTRACT

In veal calf production plant-based proteins are frequently included in milk replacer fed to the animals. Since soy products, which are mostly used, are known for their high levels of phyto-oestrogens, the effects of these feeds on the veal calf prostate were examined. Goal was to determine whether these compounds could interfere with histological screening for oestrogenic growth promoters. In a feeding experiment, four groups of veal calves fed plant-based protein-supplemented milk replacer (PBM), containing 5% soy concentrate, 5% soy isolate, 5% wheat gluten and 2% potato protein, for 4 weeks were compared to animals fed dairy-based control feed (DBM); animals treated with estradiol benzoate, diethylstilbestrol and ethinylestradiol served as positive controls. Daidzein and genistein levels measured in feed and urine showed high levels of genistein and daidzein in the soy isolate and soy concentrate supplemented feeds. Genistein and daidzein were also found in the urine of the animals that were fed these feeds. Haematoxylin–eosin-stained prostate sections of PBM-fed animals showed slight hyperplasia and some dilated tubules as compared to the DBM-fed group, but no metaplasia, which is used for screening for oestrogenic hormones. The positive controls showed extensive squamous metaplasia. Immunohistochemical staining for cytokeratin 5 (using RCK 103 monoclonal antibody) in basal cells showed a normal staining pattern of basal cells in the DBM-fed calves and extensive basal cell proliferation and squamous metaplasia in the oestrogen-treated positive control animals. PBM-fed calves showed no increase of basal cell staining but showed elongations of the basal cells in most animals, sometimes resulting in circular figures. It is concluded that the feeds examined in this study did not interfere with histological screening for oestrogens in male veal calves.

Keywords: histological screening, hormones, oestrogens, phyto-oestrogens, prostate gland, veal calves

Abbreviations: DBM, dairy-based control feed; GC-MS, gas chromatography–mass spectrometry; HE, haematoxylin and eosin; HPLC, high-performance liquid chromatography; MAT, matairesinol; ORRA, oestrogen radioreceptor assay; PBM, plant-based protein-supplemented milk replacer; SECO, secoisolariciresinol

INTRODUCTION

The use of growth promoters for fattening purposes in cattle has been banned in the European Union since 1988. In the National Plans of EU member states as required by 96/23/EC, monitoring is typically based on a list of specific analytes only; i.e., samples are checked for the absence of specific residues of growth promoters. A more effective enforcement of the 96/22/EC ban requires analysis methods designed for the screening and identification of all substances with hormonal activity.

Apart from desired effects such as increased muscle development and reduced fat deposits, anabolic agents used in animal production have been shown to produce serious changes in organs that are physiologically target tissues for these agents (Kroes, 1970; Grandmontagne, 1986; Groot *et al*., 1998). Oestrogenic hormones induce characteristic

lesions consisting of squamous metaplasia in the glandular tissue of the prostate and bulbourethral glands of male animals and in Bartholin's glands in females (Kroes, 1970; Gropp *et al*., 1976; Schaudinn and Beck, 1977; Grandmontagne, 1986; Deschamps *et al*., 1987; Rosmini, 1987; Groot *et al*., 1989; Girardi *et al*., 1990; Groot en den Hartog, 1990; Biolatti *et al*., 1994; Finazzi *et al*., 1993; Schilt *et al*., 1998; Groot *et al*., 2000).

The National Inspection Service for Livestock and Meat in The Netherlands performs histological screening for oestrogenic growth promoters in veal calves. The haematoxylin–eosin (HE) stained sections of the prostate (in male animals) and Bartholin's gland (in female animals) are examined for squamous metaplasia. This method is also used in the Regio Piemontese in Italy, and recently the supermarket chain, COOP, adopted this screening method for its own quality control programme (Biolatti *et al*., 2003).

Phyto-oestrogens are plant-derived compounds with oestrogenic actions (Lindner, 1976; Adams, 1989; Mazur and Adlercreutz, 1998; Dixon, 2004). Although their oestrogenic action is very weak as compared to natural estradiol, they can be present in high levels and may lead to oestrogenic effects in animals (Adams, 1989). The most important oestrogenic compounds in plants are isoflavones, coumestanes and lignans (Mazur and Adlercreutz, 1998). Phyto-oestrogens mimic the actions of estradiol but their effects are not identical; there are no reliable reports of phyto-oestrogens causing behavioural oestrus and their action can also be anti-oestrogenic (Adams, 1995).

In Australia and New Zealand, several cases of reduced fertility in cattle and sheep have been described as a result of grazing on phyto-oestrogen-containing clover fields or feeding of oestrogenic forage (Smith *et al*. 1979; Adams, 1995). Clinical effects were impaired ovarian function, reduced conception rates and increased embryonic loss. Phyto-oestrogens also caused signs of hyperoestrogenism such as mammary development, swelling of the vulva, discharge of cervical mucus, enlargement of the uterus and cystic ovaries (Adler and Trainin, 1960).

In sheep, phyto-oestrogens induced changes in the cervical epithelium, which showed endometrium-like epithelium in affected animals. These effects are used as a histological screen for oestrogenic effects in Australia (Adams, 1995). Phyto-oestrogens had little effect on male ruminants, but in castrated males udder development and enlargement of the bulbourethral gland were reported (Adams, 1995). In Europe, fertility disorders due to phyto-oestrogens in cattle are not often reported (Lotthammer *et al*., 1970; Khodabandehlou *et al*., 1997) and are only suggested as possible cause for the observed fertility disorders.

In milk replacers for veal calves, skim milk powder is increasingly being replaced by plant protein sources, including soybean, wheat, corn and potato (Toullec and Lallès, 1995). In soy, the oestrogenic isoflavones genistein and daidzein are most abundant and levels of 20 000–160 000 μg/100 g dry weight are reported (Mazur and Adlercreutz, 1998; Barnes *et al*., 1998). Wheat and potato contain low levels of the lignan secoisolariciresinol (SECO) (Mazur and Adlercreutz, 1998), while daidzein and genistein are too low to measure (Mazur and Adlercreutz, 1998). Table I shows the levels of daidzein, genistein and SECO as reported in literature.

It was hypothesized that these compounds as constituents of the feed may influence the histology of the target tissues, interfering with histological screening for oestrogens. The aim of this study was to investigate whether feed effects related to phyto-oestrogens might influence prostate histology in veal calves. We compared veal calves fed for 4 weeks with

TABLE I
Levels of phyto-oestrogens in ppm dry weight (Mazur and Adlercreutz, 1998)

Feed	Daidzein	Genistein	SECO	MAT
Soybean	105–560	268–841	0.13–2.73	Trace
Wheat	0	0	0.33	0.03
Potato	0	0	0.10	0.06

milk replacer containing different plant-based proteins with calves treated with estradiol benozate, ethinylestradiol and diethylstibestrol and with control animals fed dairy-based milk replacer.

MATERIALS AND METHODS

Animal material was derived from a feeding experiment and from an oestrogenic hormone experiment. The feeding experiment consisted of five groups of 8–9 male veal calves. Group 1: dairy-based control feed ($n = 8$); group 2: 5% soy concentrate (Sojcomill, 5%, $n = 9$); group 3: 5% soy isolate (Nurish, 5%, $n = 9$); group 4: 5% wheat gluten protein (Kalpro 5%, $n = 9$); group 5: 2% potato protein (Mysamine 2%, $n = 9$). (The percentages are based on dry matter of the milk replacer.)

The animals were fed dairy-based milk replacer until 20 weeks of age. The experimental feeds were given for 28 days, from week 20 to week 24. The animals were then put back onto dairy-based milk replacer and the animals were slaughtered 2 weeks later, at 26 weeks of age. The control animals were fed dairy-based feed during the whole period.

For reference purposes, three positive controls from animal experiments with oestrogens were used: one diethylstilbestrol-treated veal calf (25 mg injected intramuscularly, slaughtered after 9 days); one calf treated with ethinylestradiol (40 mg injected intramuscularly, slaughtered after 9 days); and one calf treated with estradiol benzoate (30 mg estradiolbenzoate injected intramuscularly at day 0 and day 14, slaughtered at day 28). The animals were milk-replacer-fed and slaughtered at similar age as those of the animals of the feeding experiment.

Prostate tissue was collected at slaughter and processed to paraffin sections. Sections were stained with HE and with RCK 103, an immunohistochemical stain for keratin 5 (clone RCK 103, 2202M103 Eurodiagnostica, Arnhem, The Netherlands) that stains basal cells, as described earlier (Groot *et al*., 2000). Examination of the sections was performed according to the forms used by the National Inspection Service for Livestock and Meat of The Netherlands. The sections were examined for oestrogenic effects such as changes in the urethra, dilated tubules, hyperplasia and metaplasia in the glandular tissue and the amount of mucinous cells. A semiquantitative scoring system was used: − (absent), + (slight), ++ (moderate) and +++ (severe). Presence of squamous metaplasia (+) is considered positive for oestrogenic hormones, whereas other parameters may indicate that an animal is suspect. RCK 103 was examined for staining of the urethra, basal cells, foci (3–5 stained cells) and metaplastic proliferations. Since RCK 103 stains basal cells, squamous metaplasia is easily observed.

Feed and urine (pooled samples from the feeding experiment) were analysed with HPLC/ORRA and HPLC/GC-MS (Arts *et al*., 1998) for daidzein and genistein. Urine was sampled before the experimental period (at 20 weeks of age), during the experiment (at 21 and 23 weeks of age) and after the experiment (at 25 weeks of age).

RESULTS

The histological findings in the prostate are presented in Table II. In brief, the DBM control calves (Figure 1) had normal prostate histology, one folded urethra, slight to moderate hyperplasia in the glandular tissue, one animal with dilated tubules, and no metaplasia. Prostate in the PBM-fed calves had similar characteristics, but more animals had folded urethra, more dilated tubules (Figure 2) were observed, and in some groups more hyperplasia was seen. In the DBM animals RCK 103 stained all layers of the urethra,

TABLE II
Effects on prostate histology of different plant proteins in the feed[a]

	Feed				
Feed	1 Control ($n = 8$)	2 Soy concentrate ($n = 9$)	3 Soy isolate ($n = 9$)	4 Wheat gluten ($n = 9$)	5 Potato protein ($n = 9$)
HE staining					
Urethra					
Thickened			1+		
Folded	1+		1+	3+	4+
Glands					
Dilated tubules	4+	5+ 4++	5+ 4++	6+ 2++	8+ 1++
Hyperplasia	3+ 1++	6+ 3++	9+	7+ 1++	6+ 3++
Metaplasia	−	−	−	−	−
Cellular development					
Many mucinous cells	1+	2+		2+	
Evaluation	8 negative	6 negative 3 suspect	9 negative	8 negative 1 suspect	6 negative 3 suspect
RCK 103					
Elongated cells	−	3+	5+	7+	5+
Foci 3–5 cells	2+	1+	4+	5+	5+
Circular figures	−	4+	2+	7+	5+
Metaplasia	−	−	−	−	−
Evaluation	8 negative	9 negative	9 negative	9 negative	9 negative

[a]A semiquantitative scoring system was used: – (absent), + (slight), ++ (moderate) and +++ (severe). The number of affected animals is given and the degree to which the feature was present

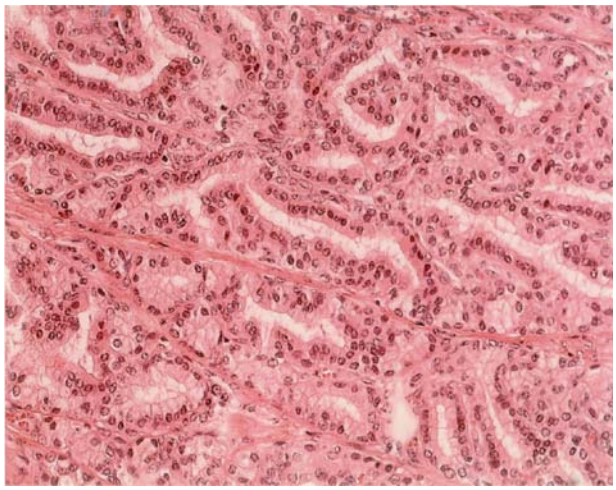

Figure 1. Normal glandular tissue of the prostate in a milk-replacer-fed veal calf. HE, ×80

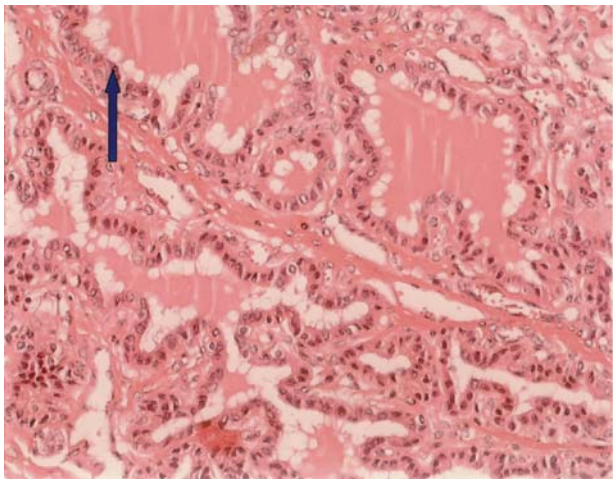

Figure 2. Glandular tissue of the prostate of a soy-concentrate-fed veal calf; note some dilated tubules with secretion (arrow). HE, ×160

scattered basal cells and excretory ducts. Some foci were observed, but no metaplasia. In the PBM groups RCK 103 stained all layers of the urethra, scattered basal cells and excretory ducts. Some animals presented with elongated cells, some animals with foci (Figure 3), and in some animals circular figures (Figure 4) were observed. None of the animals showed metaplasia.

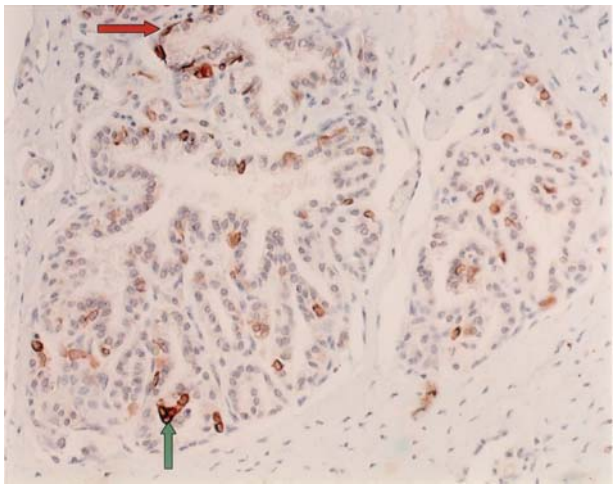

Figure 3. Basal cell staining pattern of a potato-protein-fed calf, foci (green arrow) and some elongated basal cells (red arrow); the right part of the picture shows the normal staining pattern of basal cells in the prostate. RCK 103, ×200

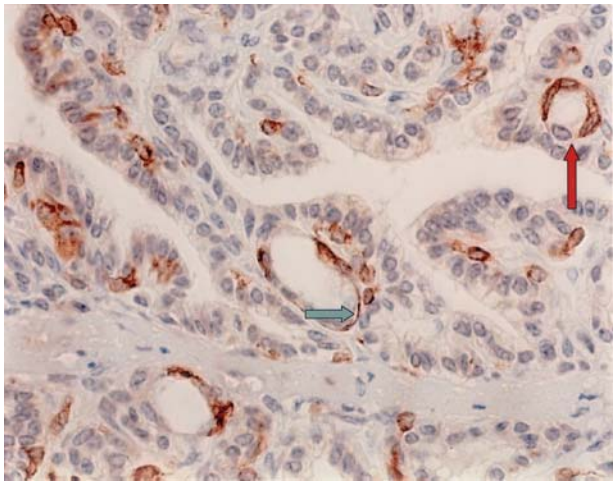

Figure 4. Elongations of basal cells (green arrow) and formation of circular figures (red arrow) in a soy-concentrate-fed veal calf. RCK 103, ×160

The estrogen-treated animals (positive controls) had the following histological changes. The diethylstilbestrol treated animal showed a thickened urethra, moderately dilated tubules in the glandular tissue with increased secretion, hyperplasia and squamous metaplasia. RCK 103 stained in the urethra the medial and basal layer, the apical layer less intensely. It also stained the basal and medial layers of the ducts, many basal cells, many foci of cells and some metaplastic proliferations, especially near the urethra.

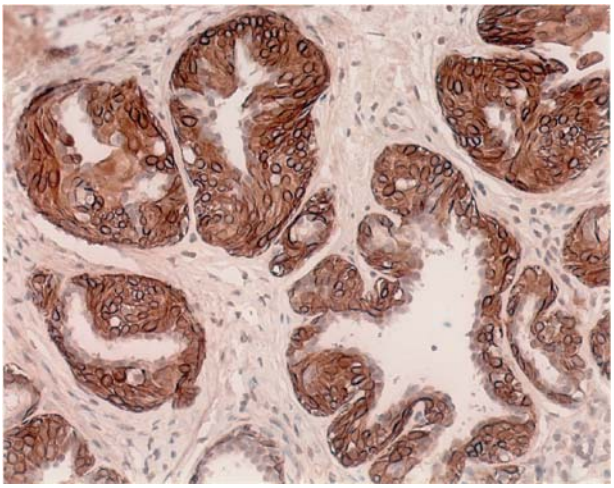

Figure 5. Basal cell proliferation leading to squamous metaplasia in an ethinylestradiol-treated veal calf. RCK 103, ×80

The ethinyloestradiol-treated animal showed a very folded urethra with thickened epithelium, slightly dilated tubules, moderate hyperplasia and extensive metaplasia (Figure 5). RCK 103 stained all layers of the urethra, all layers of the ducts, many basal cells, many foci of cells and many metaplastic proliferations.

The estradiol benzoate-treated calf had a slightly thickened and folded urethra, many dilated tubules with extensive secretion, moderate hyperplasia and metaplasia. RCK 103 stained all layers of the urethra, all layers of the ducts, many basal cells, many foci of cells and many metaplastic proliferations (Figure 6). The histological results of the oestrogen-treated calves are listed in Table III.

The levels of the main phyto-oestrogens genistein and daidzein in feed and urine are listed in Tables IV, V and VI.

DISCUSSION

The object of this study was to investigate whether phyto-oestrogen-containing feed could influence prostate histology and so interfere with histological screening for hormones. Effects of phytoestrogens in cattle have been described for animals grazing on subterranean clover in Australia (Adams, 1995). In Europe, only few cases of feed-derived oestrogenic effects in cattle have been reported (Lotthammer *et al*., 1970; Khodabandehlou *et al*., 1997), both concerning adult cattle showing fertility disorders. Effects of phyto-oestrogens in veal calves have not been described so far.

Veal calves are histologically screened for the presence of squamous metaplasia as an indication for treatment with oestrogens (Kroes *et al*., 1975). Phyto-oestrogens are reported

TABLE III
Prostate histology of oestrogen-positive control calves[a]

Feed	Diethylstibestrol ($n = 1$)	Ethinylestradiol ($n = 1$)	Estradiol benzoate ($n = 1$)
HE staining			
Urethra			
Thickened	++	++	+
Folded	−	++	++
Glands			
Dilated tubules	++	+	+++
Hyperplasia	++	++	++
Metaplasia	++	++	+
Cellular development			
Many mucinous cells	+	+	+++
Evaluation	Positive	Positive	Positive
RCK 103			
Elongated cells	−	−	−
Foci 3–5 cells	+	++	+
Metaplasia	++	+++	+++
Evaluation	Positive	Positive	Positive

[a]A semiquantitative scoring system was used: – (absent), + (slight), ++ (moderate) and +++ (severe)

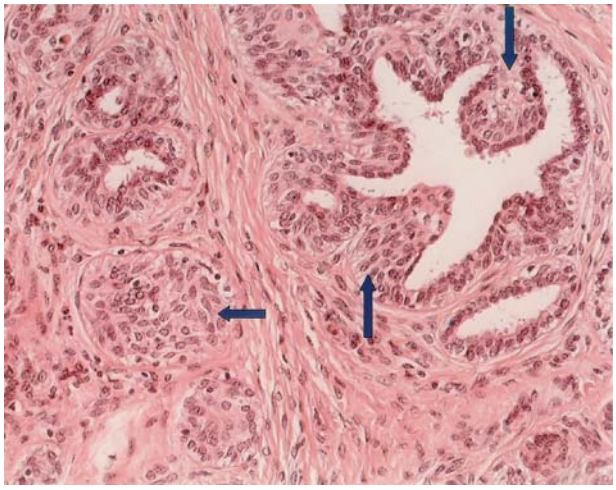

Figure 6. Squamous metaplasia (arrows) in an estradiol benzoate-treated veal calf. HE, ×160

TABLE IV
Levels of daidzein (ppm) and genistein (ppm) in the experimental feed as analysed by TNO

Feed	Daidzein	Genistein
1 (dairy based milk replacer)	<0.1	0.1–0.3
2 (5% soy concentrate)	60	80
3 (5% soy-isolate)	790	1000
4 (5% wheat gluten)	<0.1	<0.1
5 (2% potato protein)	<0.1	<0.1

TABLE V
Levels of daidzein (ppm) in urine before (week 20), during (weeks 21 and 23) and after (week 25) the experimental period

	Time in weeks of age			
Feed	20	21	23	25
1 (dairy based milk replacer)	8.3	12.0	6.8	12.0
2 (5% soy concentrate)	5.4	7.8	8.0	8.7
3 (5% soy-isolate)	12.0	5.2	7.0	13.0
4 (5% wheat gluten)	7.2	6.4	11.0	14.0
5 (2% potato protein)	8.9	4.9	7.3	15.0

TABLE VI
Levels of genistein (ppm) in urine before (week 20), during (weeks 21 and 23) and after (week 25) the experimental period

	Time in weeks of age			
Feed	20	21	23	25
1 (dairy based milk replacer)	0.3	1.0	0.2	1.1
2 (5% soy concentrate)	0.2	2.3	3.2	3.5
3 (5% soy-isolate)	0.2	8.2	14.0	15.0
4 (5% wheat gluten)	0.1	<0.1	3.3	0.4
5 (2% potato protein)	<0.1	0.2	0.9	0.5

to induce squamous metaplasia only in castrated male sheep (wethers), whereas entire male sheep are unaffected (Adams, 1995).

Veal calves are fed with milk replacers, but as well as dairy-based proteins vegetable proteins such as soy, wheat and potato protein are also included. Moreover, in recent years animals have been fed limited amounts of roughage (maize), for animal welfare reasons.

The use of plant protein in milk replacer is limited by the induction of negative side-effects in technical results due to reduced digestibility of amino acids (Branco-Pardal *et al*., 1995; Montagne *et al*., 2003) and fat (Yuangklang *et al*., 2004).

In this study, veal calves fed plant-based protein-supplemented milk replacer showed slight changes in the prostate consisting of some hyperplasia and some dilated tubules (Figure 2). Since only the soy concentrate and soy isolate supplemented feed showed high levels of daidzein and genistein, only the animals fed these feeds can be considered as phyto-oestrogen fed (Table IV). These animals also showed residues of daidzein and genistein in the urine (Tables V and VI), indicating that the phyto-oestrogens were absorbed from the feed. Low levels of SECO could be expected in the potato protein and wheat gluten fed animals (Mazur and Adlercreutz, 1998), but unfortunately these levels were not measured. The mammalian lignans enterolactone and enterodiol are products of colonic bacterial metabolism of the plant lignans mataires inol (MAT) and SECO (Axelson *et al*., 1982). In the yeast bioassay, however, enterolactone and enterodiol showed no affinity for the oestrogen receptor (Bovee *et al*., 2004), so little oestrogenic activity was to be expected.

For screening as performed by the National Inspection Service, the results mean that in this study all control animals were negative and showed normal prostate histology as described earlier (Groot and Biolatti, 2004). From the 18 phyto-oestrogen-fed animals, 3 animals (16%) would be suspected of hormonal influence, whereas none of the animals would be judged as positive. Animals are considered positive only when they show squamous metaplasia in the glandular tissue, and such animals or the farms from which they come are further investigated for residues of hormones.

Phyto-oestrogen-containing feed did not affect the screening for oestrogenic hormones in this study. Moreover, changes in the phyto-oestrogen-fed animals were comparable to those of the other groups of animals fed PBM (Table II), in which no phyto-oestrogens were detected, and differed only slightly form the control group. The differences from the control group were only quantitative and may be regarded as part of the natural variation in control animals. Differences between the experimental animals and the positive controls (Table III), however, were striking (Figures 4 and 5). It appears that in this study no specific phyto-oestrogen effects could be discerned in the prostate.

The RCK 103 staining showed some interesting changes in the PBM-fed animals as compared to the negative and positive controls. In normal animals, RCK 103 stains basal cells and multilayered epithelium such as the urethra and the excretory ducts in the calf prostate. In the oestrogen-treated animals there is a marked basal cell proliferation leading to increased staining of the glandular tissue and marked staining of squamous metaplasia (Groot *et al*., 2000). In the PBM animals, however, there was no increase of basal cell staining and no squamous metaplasia, but some basal cells showed cytoplasmatic elongations, which may form circular figures. The significance of these figures is not known. This staining indicated that PBM, with or without phyto-oestrogens, did not induce basal cell proliferation as oestrogens do but may have more subtle effects on the basal cells in the prostate.

Female veal calves are seldom used for animal experiments, so the effects of phyto-oestrogens on the Bartholin's gland still have to be investigated. In practice, however, most veal calves (>90 %) are male.

It is concluded that, although phyto-oestrogen-containing feeds may have a slight influence on prostate histology, in the feeds tested this does not interfere with the screening used for control of oestrogenic growth promoters in male veal calves.

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